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## Increased Virulence of a Fibronectin-Binding Protein Mutant of *Staphylococcus aureus* in a Rat Model of Pneumonia

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**Fibronectin-binding proteins mediate *Staphylococcus aureus* internalization into nonphagocytic cells in vitro. We have investigated whether fibronectin-binding proteins are virulence factors in the pathogenesis of pneumonia by using *S. aureus* strain 8325-4 and isogenic mutants in which fibronectin-binding proteins were either deleted (DU5883) or overexpressed [DU5883(pFnBPA4)]. We first demonstrated that fibronectin-binding proteins mediate *S. aureus* internalization into alveolar epithelial cells in vitro and that *S. aureus* internalization into alveolar epithelial cells requires actin rearrangement and protein kinase activity. Second, we established a rat model of *S. aureus*-induced pneumonia and measured lung injury and bacterial survival at 24 and 96 h postinoculation. *S. aureus* growth and the extent of lung injury were both increased in rats inoculated with the deletion mutant (DU5883) in comparison with rats inoculated with the wild-type (8325-4) and the fibronectin-binding protein-overexpressing strain DU5883(pFnBPA4) at 24 h postinfection. Morphological evaluation of infected lungs at the light and electron microscopic levels demonstrated that *S. aureus* was present within neutrophils from both 8325-4- and DU5883-inoculated lungs. Our data suggest that fibronectin-binding protein-mediated internalization into alveolar epithelial cells is not a virulence mechanism in a rat model of pneumonia. Instead, our data suggest that fibronectin-binding proteins decrease the virulence of *S. aureus* in pneumonia.**

In a recent survey, *Staphylococcus aureus* was found to be the most common cause of lower respiratory tract infections in Europe, the United States, Canada, Latin America, and the Western Pacific region (9). In view of the fact that *S. aureus* infections are increasingly difficult to treat because of the high percentage of antibiotic-resistant strains (34), a better understanding of the molecular basis of *S. aureus* virulence in pneumonia may help in the design of new therapeutic strategies.

*S. aureus* has long been regarded as an extracellular pathogen because it is rarely observed inside cells in vivo and because it secretes a range of toxins that are cytolytic to many host cell types (14, 29). However, recent in vitro studies demonstrate that *S. aureus* is internalized and survives inside nonphagocytic cells (1, 2, 12, 18, 19, 21). Fibronectin-binding proteins present on the surface of *S. aureus* (16, 19, 26) mediate internalization into nonphagocytic cells. *S. aureus* fibronectin-binding proteins bind  $\beta$ 1-integrins on the surface of the host cells by means of a fibronectin bridge (16).

Survival of internalized *S. aureus* within nonphagocytic cells may be an additional virulence mechanism in *S. aureus* infections (20). Internalized *S. aureus* may be able to evade or delay elimination by the host's immune system and avoid extracellular antibiotics (20). If internalization contributes to *S. aureus* persistence in vivo, then drugs which interfere with fibronectin binding to host cell integrins may have a role to play in treatment of *S. aureus* infections (6).

Alveolar epithelial type I cells are large squamous cells that

cover over 95% of the lungs' surface area; the remaining 5% is covered by alveolar epithelial type II cells. Both alveolar epithelial type I and II cells have a number of potential fibronectin-binding receptors on their cell surfaces (7, 28, 32). The overall objective of our study was to investigate whether fibronectin-binding protein-mediated internalization into alveolar epithelial cells is a virulence mechanism in *S. aureus*-induced pneumonia.

### MATERIALS AND METHODS

All reagents and materials were supplied by Sigma, Dorset, United Kingdom, unless stated otherwise.

**Bacteria.** The *S. aureus* strains used in this study are derivatives of the wild-type strain 8325-4. Strain DU5883 is an isogenic mutant of strain 8325-4 disrupted in the *fnbA* (*fnbA::Tc<sup>r</sup>*) and *fnbB* (*fnbB::Em<sup>r</sup>*) genes (17). DU5883(pFnBPA4) is the deletion mutant with *fnbA* expressed on a high copy plasmid (17). All strains were grown overnight in Todd Hewitt broth (B. D. Biosciences, Oxford, United Kingdom); DU5883(pFnBPA4) was selected with 10  $\mu$ g of chloramphenicol per ml. The identity of each *S. aureus* strain was regularly checked by using antibiotic disks (B. D. Biosciences). Overnight cultures were washed twice with endotoxin-free phosphate-buffered saline (PBS) before resuspension in PBS for all experiments.

**Alveolar epithelial cell line.** Simian virus 40 (SV40)-transformed strain AT2 neonatal alveolar epithelial cells were used for the in vitro internalization assays (4). SV40-AT2 cells retain the sodium transport properties of alveolar type II cells and express RTI<sub>40</sub> (rat alveolar epithelial type I cell protein; molecular mass, approximately 40 kDa) (25, 31). SV40-AT2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Labtech International, East Sussex, United Kingdom), penicillin (100 U/ml), and streptomycin sulfate (100  $\mu$ g/ml) (Invitrogen, Paisley, United Kingdom). SV40-AT2 cells were maintained at 37°C in a 5% CO<sub>2</sub> humidified incubator (31). SV40-AT2 cells were prepared for internalization assays by seeding semiconfluent 75-cm<sup>2</sup> flasks onto six-well plates (Fred Baker Scientific, Cheshire, United Kingdom) and incubating overnight in DMEM plus 10% FCS.

Control SV40-AT2 cells were checked during the experimental period for mycoplasmas by immunofluorescence staining with Hoechst 33258 DNA-binding

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dye (only the SV40-AT2 nuclei stained with the DNA-binding dye in mycoplasma-negative cells) (38).

**Internalization assay.** Confluent SV40-AT2 cells were incubated for 1 h in serum-free DMEM and then washed twice in PBS with calcium and magnesium. DMEM (1 ml) containing  $10^6$  CFU of *S. aureus* per ml was added to each well that contained SV40-AT2 cells (approximately  $1.5 \times 10^6$  SV40-AT2 cells per well). *S. aureus* was cocultured with SV40-AT2 cells for 2 to 6 h. At the end of the coculture period, SV40-AT2 cells were washed twice with PBS and then incubated for 1 h in the presence of gentamicin (100  $\mu$ g/ml in serum-free DMEM). The SV40-AT2 cells were then washed three times with PBS and lysed with 1% (wt/vol) NP-40 (ICN Biomedicals, Basingstoke, United Kingdom) in 10 mM Tris-HCl buffer, pH 8.0, containing 154 mM NaCl and complete protease inhibitor cocktail (Roche Diagnostics, East Sussex, United Kingdom). The cell lysate was diluted and plated out in triplicate on tryptic soy agar (TSA) plates supplemented with sheep blood (B. D. Biosciences). The plates were incubated overnight at 37°C, and the number of CFU was recorded to determine the number of intracellular *S. aureus*. *S. aureus* CFU were only recovered in the presence of SV40-AT2 cells.

**Effects of cytochalasin D and genistein.** The cytochalasin D and genistein assay was a modification of the internalization assay described above. SV40-AT2 cells were incubated for 1 h at 37°C with either cytochalasin D (2  $\mu$ M) or genistein (250  $\mu$ M) (Roche Diagnostics) (18). Internalization assays were then performed in the presence of inhibitors for 2 h at 37°C. At the end of the experimental period, SV40-AT2 cells were washed and incubated with gentamicin. In some experiments, washed SV40-AT2 cells were reincubated with *S. aureus* for an additional 2 or 4 h to demonstrate that cytochalasin D and genistein were not toxic to SV40-AT2 cells. The effects of cytochalasin D and genistein on the number of internalized *S. aureus* were compared to the extent of *S. aureus* internalized in the absence of inhibitors.

**Pneumonia model.** Male specific-pathogen-free rats (300 to 350 g) were obtained from Harlan United Kingdom, Ltd. (Oxton, United Kingdom). Rats were anaesthetized with an intraperitoneal injection of 1 ml of a mixture of Hypnorm (0.315 mg of fentanyl citrate and 10 mg of fluanisone per ml) and Hypnoval (5 mg of midazolam hydrochloride per ml) (40). The rat's mouth was opened, and the trachea was visualized with the aid of a laryngoscope. A catheter (0.96 cm external diameter, 9 cm in length) (Portex fine bore polyethylene tubing; Portex Ltd., Kent, United Kingdom) was inserted into the distal airways. *S. aureus* (approximately  $10^8$  CFU/0.5 ml) was instilled into the distal airways down the catheter. PBS was instilled into the distal airways of control rats.

**Experimental design.** Lung tissue was obtained from rats inoculated with 8325-4 ( $n = 3$ ), DU5883 ( $n = 3$ ), or PBS (vehicle) ( $n = 3$ ) at 24 h postinfection for analysis at the light microscopic level. For morphological analysis of lung tissue at the electron microscopic level, lung tissue was obtained from rats inoculated with 8325-4 ( $n = 2$ ), DU5883 ( $n = 2$ ), and PBS ( $n = 2$ ) at 24 h postinfection. Bronchoalveolar lavage (BAL) fluid and lung tissue were obtained from rats inoculated with 8325-4 at 24 h ( $n = 7$ ) and 96 h ( $n = 5$ ) postinfection; from rats inoculated with DU5883 at 24 h ( $n = 7$ ) and 96 h ( $n = 6$ ) postinfection; and from rats inoculated with DU5883(pFnBPA4) at 24 h ( $n = 7$ ) and 96 h ( $n = 5$ ) postinfection. Control rats (PBS instilled) were also included at 24 h ( $n = 7$ ) and 96 h ( $n = 5$ ). All animal experiments were carried out according to the Animals (Scientific Procedures) Act, 1986.

**Collection of lung tissue, blood, and BAL fluid samples.** At the end of the experimental period, rats were anaesthetized with pentobarbital (45 mg/kg intraperitoneally) containing heparin (500 U/kg intraperitoneally) (22–24). Blood was collected from the ascending aorta. The trachea was cannulated, and the lungs were lavaged two times with sterile PBS. The lungs were then removed. BAL fluid was centrifuged at  $900 \times g$  for 5 min (23, 24). BAL fluid leukocytes were quantified by hemacytometric counting. Differential cell counts on BAL fluid cells were performed on cytocentrifuged preparations fixed with methanol and stained with Diff-Quick. A total of 300 cells were counted per cytospin.

**Protein assay.** BAL fluid protein concentration was determined by using the Bio-Rad assay (Bio-Rad Laboratories, Hertfordshire, United Kingdom) with bovine serum albumin as a standard (Pierce Warriner, Chester, United Kingdom) (24). Data are expressed as the total amount of protein recovered in BAL fluid (i.e., milligrams of protein per milliliter multiplied by the total volume of BAL fluid recovered).

**RTI<sub>40</sub> assay.** The amount of RTI<sub>40</sub> (a biochemical marker for type I cell damage) was measured in an enzyme-linked immunosorbent assay (ELISA)-based assay essentially as described previously (22, 23). RTI<sub>40</sub> was assayed on the insoluble fraction of BAL fluid ( $500,000 \times g_{av}$  for 10 min in a Beckman Optima ultracentrifuge). Data are calculated as the total amount of RTI<sub>40</sub> recovered in BAL fluid (i.e., relative densitometry units of RTI<sub>40</sub> per milliliter multiplied by

the total volume of BAL fluid) and then expressed as a percentage of the control values.

**Lung homogenates.** Lungs were weighed and homogenized in Tris-HCl (2.42 mM)-buffered saline (TBS), pH 8.2, containing NaCl (154 mM) and proteinase inhibitor cocktail (Roche Diagnostics). Lungs were homogenized by using a blender (Ultra Turrax Y25; IKA-Werke) on ice at approximately 4,000 rpm twice for 5 s each. Lung homogenates were serially diluted and plated out on blood agar plates to determine the CFU.

**Immunofluorescence analysis.** Lungs were fixed with paraformaldehyde (4% [wt/vol] in PBS) to determine the extent of alveolar epithelial necrosis in *S. aureus* pneumonia at 24 h postinfection (24). Frozen lung sections (3  $\mu$ m) were incubated with monoclonal antibodies (MAb) against alveolar epithelial type I cells (anti-RTI<sub>40</sub> MAb) (10) and two different MAb against alveolar epithelial type II cells (anti-MMC4 antigen and anti-RTII<sub>70</sub>) (3) (11), followed by isotype-specific secondary antibodies. Fluorescein isothiocyanate (FITC) conjugated to anti-immunoglobulin G1 (IgG1) was used to detect the RTI<sub>40</sub> protein (Lorne Laboratories, Reading, United Kingdom) (3), Rhodamine-conjugated to anti-IgG2a (Lorne Laboratories) was used to detect the MMC4 protein (3), and Alexa Fluor 350 conjugated to anti-IgG3 was used to detect the anti-RTII<sub>70</sub> antigen (Molecular Probes, Lieden, Netherlands). The nucleus was stained with the DNA-binding dye Hoechst 33258. Immunofluorescence-stained sections were observed by epifluorescence illumination (Axiovert S100; Zeiss). Images were captured with a Cool Snap camera, and the images were processed by using Improvision OpenLab software (version 2.2).

**Gram stain.** Paraffin sections were Gram stained to visualize *S. aureus* in lung tissue at 24 h postinfection. Cytospun cells were also Gram stained to identify *S. aureus* in neutrophils and macrophages from BAL fluid.

**Electron microscopic analysis.** Tissue samples for electron microscopy were selected from the hemorrhaged region of lungs inoculated with either 8325-4 or DU5883. Similar regions were taken from control lungs. Lung blocks were fixed with glutaraldehyde buffer (3%) containing sodium cacodylate (0.1 M). Lung blocks were postfixed in osmium tetroxide buffer (1%) containing sodium cacodylate (0.1 M). Lung blocks were then dehydrated in a graded series of alcohol and embedded in polyethylene capsules in fresh Araldite epoxy resin. Lung sections were cut and placed on 200-mesh uncoated grids and then stained with uranyl acetate and lead citrate for electron microscopic studies. Sections were viewed with a Philips CM12 electron microscope.

**Statistical analysis.** All data are expressed as means  $\pm$  standard error of the mean. Data from the in vitro internalization assays were transformed to reflect the total number of *S. aureus* recovered per well or transformed to fractions of control values. Multiple sample means were compared by using one-way analysis of variance with the Student-Newman-Keuls posttest.

Multiple sample means from data generated from the pneumonia models (CFU, leukocyte numbers, and BAL protein and RTI<sub>40</sub> concentrations) were compared by using Kruskal-Wallis analysis of variance (nonparametric analysis of variance), with Dunn's multiple comparison posttest.  $P < 0.05$  was considered significant. Tests were performed by using GraphPad InStat v3.00 for Windows 95 (www.graphpad.com).

## RESULTS

**Fibronectin-binding proteins are important for the internalization of *S. aureus* by SV40-AT2 cells.** The presence or absence of fibronectin-binding proteins (Fig. 1) significantly affected the extent of *S. aureus* internalization into SV40-AT2 cells. DU5883, which does not express either FnBPA or -B, was internalized 20-fold less than 8325-4 ( $P < 0.01$ ). DU5883(pFnBPA4), which overexpresses FnBPA, was internalized 2.4-fold more than 8325-4 ( $P < 0.001$ ) (Fig. 1). These data demonstrate that fibronectin-binding proteins are important for internalization of *S. aureus* into rat alveolar epithelial cells.

**SV40-AT2 intracellular traffic is important for internalization of *S. aureus*.** *S. aureus* strain 8325-4 was cocultured with SV40-AT2 cells for 2 h in the presence of inhibitors of intracellular traffic (Fig. 2). Cytochalasin D, an inhibitor of actin polymerization, reduced the extent of *S. aureus* internalization by 91% (Fig. 2). Genistein, a protein tyrosine kinase inhibitor, reduced internalization by 74% ( $P < 0.01$ ) (Fig. 2). The inhib-

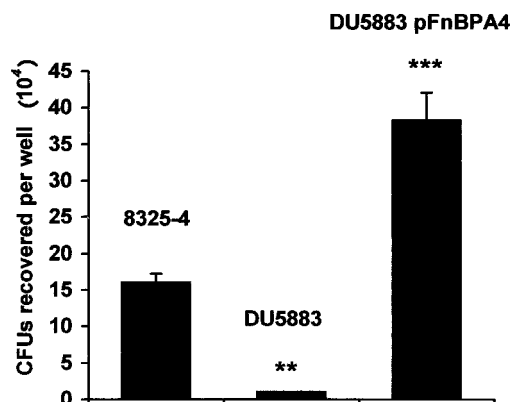


FIG. 1. Effect of fibronectin-binding proteins on *S. aureus* internalization into alveolar epithelial cells. SV40-AT2 cells were cocultured with either 8325-4, DU5883, or DU5883(pFnBPA4) for 6 h. Extracellular *S. aureus* cells were killed with gentamicin. The experiment was performed three times; data from one representative experiment are shown. \*\*,  $P < 0.01$  versus control; \*\*\*,  $P < 0.001$  versus control.

itors were not toxic to SV40-AT2 cells, since the extent of internalization increased following the removal of these inhibitors from the incubation medium (Fig. 2).

**Morphological changes to the lung in 8325-4-infected rats.** Distal airway instillation of 8325-4 into rat lungs induced pneumonia in the left lung in the majority of experiments. At autopsy, the involved lobe turned from red at 24 h postinfection to grey at 96 h postinfection. Light microscopic analysis of lung tissue at 24 h postinfection demonstrated that leukocytes had filled the distal air spaces (Fig. 3C).

The morphological integrity of alveolar epithelial type I and II cells in 8325-4-infected lungs was determined by immuno-

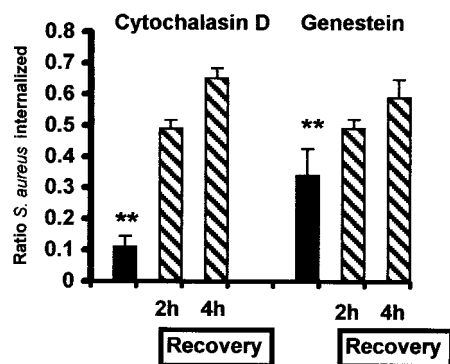


FIG. 2. Effects of cytochalasin D and genistein on *S. aureus* internalization into alveolar epithelial cells. SV40-AT2 cells were cocultured with 8325-4 in the presence of inhibitors for 2 h (black bars). SV40-AT2 cells were then washed and reincubated for an additional 2 h or 4 h (diagonal stripes) in the presence of only *S. aureus*. The extent of internalization was compared at each time point with that of SV40-AT2 cells cultured with *S. aureus* alone. Data are presented as the ratio of *S. aureus* internalized in the presence of inhibitor to *S. aureus* internalized in the absence of inhibitor. The experiment was performed twice with similar results. Data from one experiment are shown. Both inhibitors significantly reduced the extent of 8325-4 internalization into SV40-AT2 cells in comparison to SV40-AT2 cells cocultured with just 8325-4. \*\*,  $P < 0.01$  versus SV40-AT2 cells cocultured with 8325-4.

fluorescence analysis with the aid of cell-selective antibodies (3, 10, 11). Type I cells were visualized with the aid of an anti-RTI<sub>40</sub> MAb (green staining) (Fig. 3 and 4). In control lungs, the anti-RTI<sub>40</sub> MAb bound to the apical surface of alveolar epithelial type I cells (Fig. 3 and 4). In 8325-4-inoculated lungs, the pattern and extent of anti-RTI<sub>40</sub> binding were similar to those in control lungs in most regions of the lung (Fig. 3 and 4).

Alveolar epithelial type II cells were identified by using two different MAbs. The anti-RTII<sub>70</sub> MAb recognizes the apical surface of type II cells (blue apical staining) (Fig. 4) (11), while the MMC4 MAb recognizes the apical surface of alveolar epithelial type II and Clara cells (red staining) (Fig. 4) (3). The pattern and extent of both anti-type II MAb binding in 8325-4-infected lungs 24 h postinfection were comparable to that in control sections (Fig. 4). Occasionally, MMC4- and RTII<sub>70</sub>-positive cells (type II cells) were rounded-up and appeared to be in the process of shedding from their basement membranes in 8325-4-infected lungs at 24 h postinfection (data not shown).

**Effect of fibronectin-binding proteins on recovery of *S. aureus* in lung tissue and BAL fluid.** The number of CFU recovered in lung tissue 24 h postinfection decreased by over 97% in rats inoculated with either 8325-4 or DU5883(pFnBPA4) in comparison to the number of CFU instilled (Fig. 5). In contrast, the number of CFU recovered in lung tissue from DU5883-inoculated rats was increased by 4.6-fold relative to the instillate at 24 h postinfection (Fig. 5). Moreover, the number of CFU recovered in DU5883-infected lungs at 24 h was increased by over 190-fold relative to the number in 8325-4-infected rats ( $P < 0.05$ ) and by 1,700-fold ( $P < 0.01$ ) in comparison to the number of CFU recovered from DU5883(pFnBPA4)-infected lungs (Fig. 5). By 96 h postinfection, the number of CFU recovered from DU5883-inoculated lungs was approaching the number of CFU obtained from both 8325-4- and DU5883(pFnBPA4)-inoculated lungs (Fig. 5).

In contrast to the lung data, the number of CFU recovered in BAL fluid at 24 h was not significantly different between the three *S. aureus* strains (Fig. 5). No CFU were recovered in BAL fluid at 96 h postinfection in any of the *S. aureus*-infected lungs.

**Effect of fibronectin-binding proteins on leukocyte recruitment to the lungs in pneumonia.** The total number of leukocytes recovered in BAL fluid from 8325-4-infected rats was elevated 57-fold above control values at 24 h (Fig. 6A). At 24 h postinfection, most of the leukocytes in BAL fluid from 8325-4-infected rats were neutrophils (90%) (Fig. 6B). However, by 96 h postinfection the percentage of neutrophils had decreased, while that of both monocytes/macrophages and lymphocytes had increased (Fig. 6B).

The total number of leukocytes recovered in BAL fluid from DU5883- and DU5883(pFnBPA4)-infected rats was significantly elevated over control values, by 37-fold and 33-fold, respectively (Fig. 6A). However, there were no significant differences in the total number of leukocytes in BAL fluid between the different strains at either 24 or 96 h postinfection. Nor was the percentage of neutrophils, macrophages/monocytes, and lymphocytes recovered in BAL fluid significantly different between *S. aureus* strains at either 24 or 96 h postinfection (data not shown).

**Damage to the air-blood barrier in *S. aureus* pneumonia.**



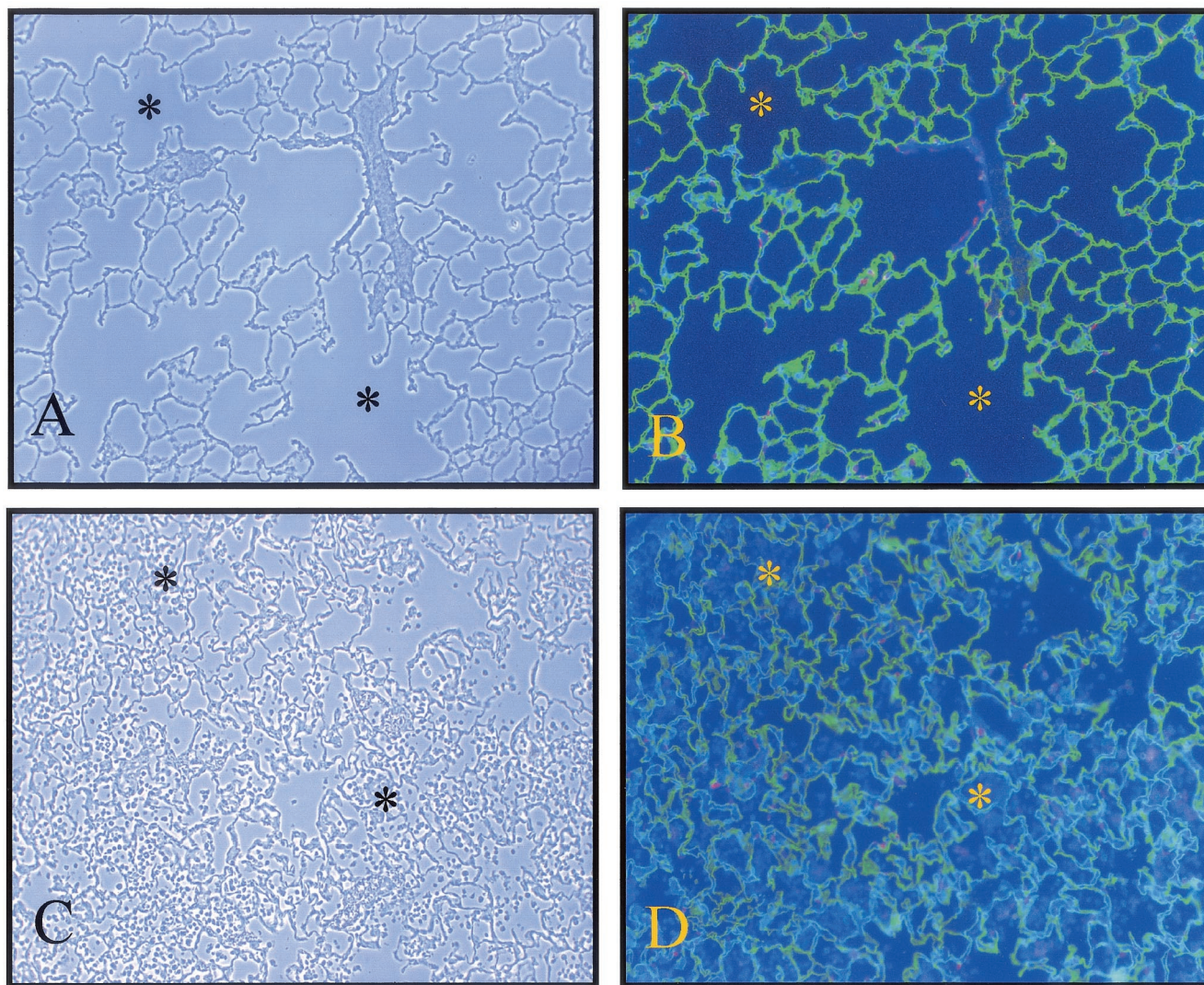


FIG. 3. Immunofluorescence visualization of leukocyte influx and alveolar epithelium in *S. aureus* 8325-4-inoculated lungs at 24 h postinfection. Alveolar epithelial type I cells were stained with the anti-RTI<sub>40</sub> MAb (green), while alveolar epithelial type II cells were stained with the MMC4 MAb (red). Nuclei were stained with the Hoechst DNA-binding dye. (A) Phase-contrast image of control lung. (B) Corresponding immunofluorescence image. (C) Phase-contrast image of 8325-4-inoculated lung. (D) Corresponding immunofluorescence image. Many of the air spaces (stars) are filled with leukocytes ((Hoechst 33258-only-positive cells) in 8325-4-inoculated lungs in comparison with control lungs. Original magnification,  $\times 100$ .

The amount of protein recovered in BAL fluid from 8325-4-infected rats was elevated by 6.8-fold above control values at 24 h postinfection but by only 2.7-fold at 96 h postinfection (Fig. 7A). The amount of protein recovered in BAL fluid from DU5883-infected rats was significantly elevated over values from both control and DU5883(pFnBPA4)-inoculated rats at 24 h postinfection (Fig. 7A). However, by 96 h postinfection, the amount of protein recovered in BAL fluid was not significantly different between the three different strains (Fig. 7A).

To determine the extent of alveolar epithelial type I cell necrosis in *S. aureus* pneumonia, the amount of RTI<sub>40</sub> was also measured in BAL fluid. The amount of RTI<sub>40</sub> recovered in BAL fluid from 8325-4- and DU5883(pFnBPA4)-infected rats was not significantly different from control values (Fig. 7B). However, the amount of RTI<sub>40</sub> recovered from rats inoculated

with DU5883 was significantly elevated over both control and DU5883(pFnBPA4)-infected rats at 24 h postinfection (Fig. 7B). There were no differences in the amount of RTI<sub>40</sub> recovered in BAL fluid from rats inoculated with *S. aureus* at 96 h postinfection (data not shown).

**Cellular location of *S. aureus* in 8325-4- and DU5883-infected lungs.** Gram stains of both BAL fluid cells (cytospins) and infected lung tissue (paraffin sections) demonstrated that *S. aureus* was predominantly associated with neutrophils from infected rats (data not shown). In addition, the percentage of neutrophils containing one or more Gram stain particles was not different between 8325-4- and DU5883-infected lungs ( $10.3 \pm 1.45$  versus  $11.8 \pm 6.08$ , respectively). Macrophages occasionally contained a few gram-positive particles in their cytoplasm. Gram-positive particles were very occasionally as-



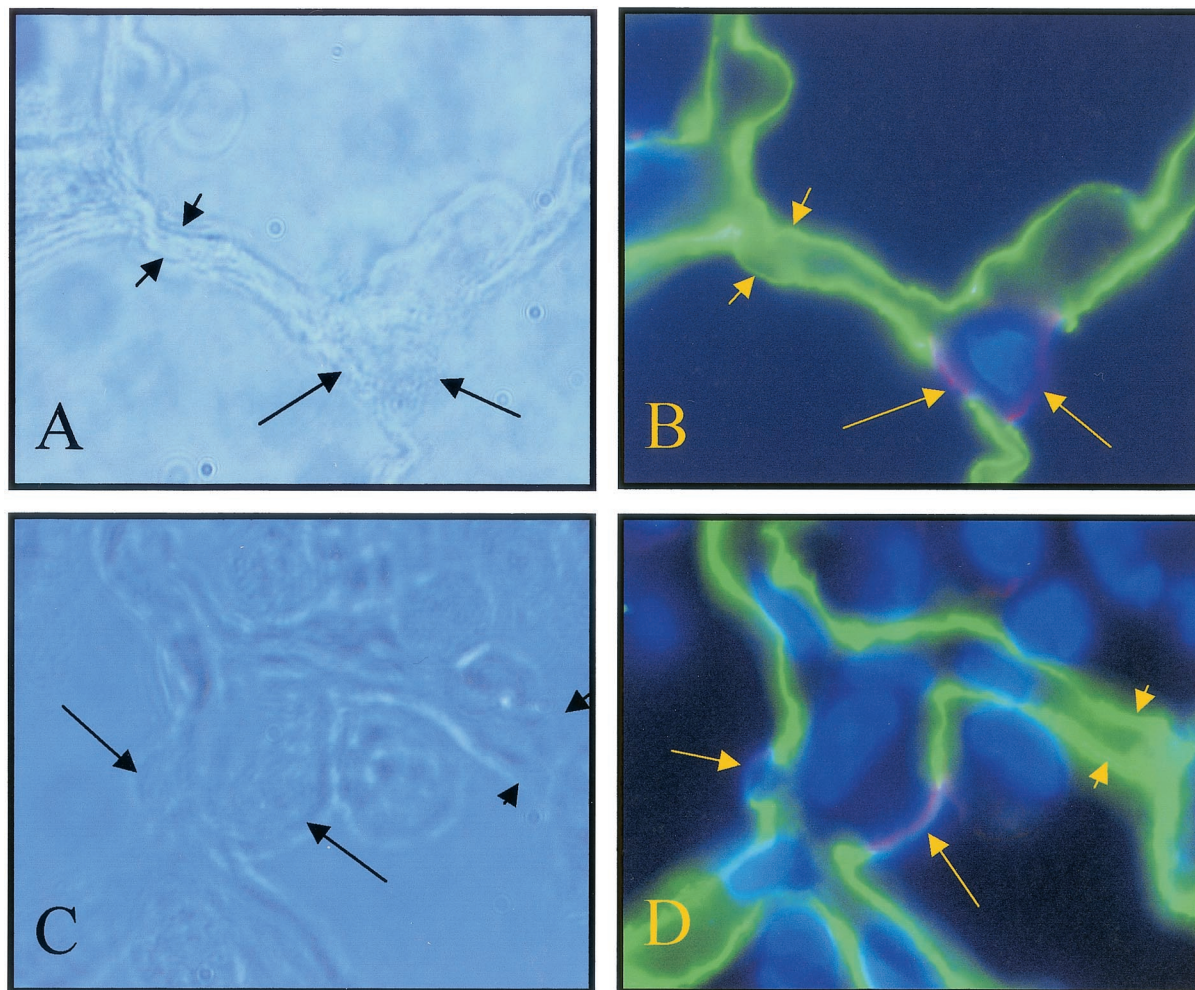


FIG. 4. High-magnification view of alveolar epithelium in *S. aureus* 8325-4-inoculated lungs at 24 h postinfection. Alveolar epithelial type I cells were stained with the anti-RTI<sub>40</sub> MAb (green, short arrows), and alveolar type II cells were stained with the MMC4 and anti-TII<sub>70</sub> cell MAb (red and blue, respectively, long arrows). Nuclei were stained with the Hoechst DNA-binding dye. (A) Phase-contrast image of control lung. (B) Corresponding immunofluorescent image. (C) Phase-contrast image of 8325-4-inoculated lung. (D) Corresponding immunofluorescent image. Binding of alveolar cell-selective antibodies in 8325-4-infected lungs was not dissimilar from that in control lungs despite the ongoing inflammation. Original magnification,  $\times 1,000$ .

sociated with the alveolar wall in both 8325-4- and DU5883-infected lungs (data not shown). However, it was not possible to resolve the location of the gram-positive particles by light microscopy.

Electron microscopic analysis confirmed that neutrophils from both 8325-4- and DU5883-infected lungs contained *S. aureus* (Fig. 8A and B). Electron microscopic analysis of lung tissue failed to demonstrate any *S. aureus* internalized with alveolar epithelial cells in 8325-4-inoculated rats. However, *S. aureus* was observed in one necrotic alveolar epithelial type II cell from DU5883-inoculated rats (Fig. 8C and D).

## DISCUSSION

We have demonstrated that *S. aureus* is internalized into rat alveolar epithelial cells in vitro and that internalization is mediated by fibronectin-binding proteins located on the surface of the bacteria. *S. aureus* internalization into alveolar epithelial

cells also requires an active cytoskeleton, since internalization was inhibited by cytochalasin D, which disrupts actin structures. Furthermore, our data suggest that *S. aureus* internalization is a regulated process, since the inhibition of tyrosine kinases by genistein prevented uptake. Similar results have been reported previously for *S. aureus* internalization into other nonprofessional phagocytic cells, including mammary gland epithelial cells, endothelial cells, and osteoblasts (1, 12, 15, 18, 24, 26).

To determine whether the expression of fibronectin-binding proteins contributes to virulence in vivo, we first established a rat model of *S. aureus*-induced pneumonia (strain 8325-4). Instillation of *S. aureus* ( $10^8$  CFU) into the distal airways of rat lungs induced an acute inflammatory reaction that was resolving by 96 h postinfection. The total number of leukocytes and the percentage of neutrophils in BAL fluid were both elevated at 24 h postinfection but had returned to near control values at 96 h postinfection. Similarly, the amount of protein recovered

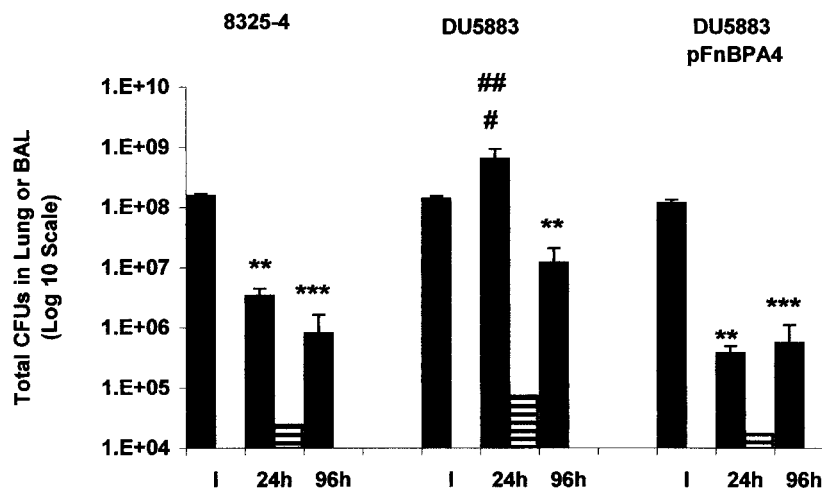


FIG. 5. Number of viable *S. aureus* recovered in lung tissue and BAL fluid at 24 and 96 h postinfection. The number of viable *S. aureus* (CFU) was determined in lung tissue (black bars) and BAL fluid (horizontal bars). \*\*,  $P < 0.01$  versus instillate CFU; \*\*\*,  $P < 0.001$  versus instillate CFU; #,  $P < 0.05$  versus 8325-4; ##,  $P < 0.01$  versus DU5883(pFnBPA4).

in BAL fluid, which is a measure of serum flux into the air spaces, was elevated at 24 h but returned to near control levels at 96 h postinfection. However, despite the elevated number of leukocytes and protein in BAL fluid, our data did not reveal major necrosis of alveolar epithelial type I cells in 8325-4-induced pneumonia.

To determine whether fibronectin-binding proteins contribute to *S. aureus* virulence in pneumonia, we compared the survival of three *S. aureus* strains. These three strains were the wild-type 8325-4, a mutant (DU5883) in which both the *fnbPA* and *fnBPB* genes were deleted, and the same mutant containing the gene for FnBPA on a high-copy-number plasmid [DU5883(pFnBPA4)] (17). Contrary to what we expected, the number of viable DU5883 cells in lung tissue at 24 h postinfection was 190-fold greater than the number of 8325-4 cells and 1,700-fold greater than the number of DU5883(pFnBPA4) cells. However, by 96 h postinfection, the number of viable DU5883 *S. aureus* had declined to values approaching those of both the wild type and the complemented mutant. These data suggest that fibronectin-binding proteins promote *S. aureus* elimination from the lungs.

The extent of acute lung injury, as measured by the total amount of protein and RTI<sub>40</sub> recovered in BAL fluid, was also increased at 24 h postinfection in DU5883-infected rats in comparison with values obtained from rats inoculated with either the parental 8325-4 or the complemented mutant strain. The amount of RTI<sub>40</sub> in BAL fluid is associated with the extent of morphological injury (necrosis) to alveolar epithelial cells in other models of acute lung injury (22–24). It is possible that the increased protein content of BAL fluid may represent increased serum flux into the air spaces secondary to necrosis of type I cells (22). Our data therefore suggest that the additional *S. aureus* burden in DU5883-infected rats increased the severity of alveolar wall injury in comparison to 8325-4- and DU5883 (pFnBPA)-inoculated lungs at 24 h postinfection. Our data demonstrate that the absence of fibronectin-binding proteins promotes alveolar wall injury in a rat model of *S. aureus* pneumonia.

Many studies demonstrate that highly encapsulated forms of bacterial pathogens, including *S. aureus*, are more virulent than the weakly encapsulated forms (5, 35, 36). This difference in virulence is thought to be due to the fact that encapsulated forms are more resistant to internalization by both professional (e.g., neutrophils) and nonprofessional (e.g., epithelial cells) phagocytes in vivo (5, 35, 36). We have not determined why strain DU5883 survives better in lung tissue than 8325-4 and DU5883(pFnBPA4). Our data demonstrate that the number of neutrophils recovered in BAL fluid at 24 h postinfection is not significantly different among the three strains. Moreover, neutrophil recruitment to lung tissue, as measured by myeloperoxidase activity, was not significantly different between 8325-4- and DU5883-infected lungs (unpublished observations).

Soluble fibronectin, which is present in BAL fluid, facilitates neutrophil phagocytosis and killing of *S. aureus* in in vitro assays (13, 30, 41). The fibronectin-binding protein-deficient *S. aureus* strain may be less efficiently cleared by neutrophils in comparison with fibronectin-binding protein-expressing strains because it does not bind fibronectin. Alternatively, fibronectin bound to fibronectin-binding protein-expressing strains of *S. aureus* may enhance alveolar macrophage activation in comparison with the knockout mutant (39). We are currently investigating these two possibilities.

The exact role of fibronectin-binding proteins in *S. aureus* virulence is unclear. On one hand, all of the clinical strains of *S. aureus* tested so far contain one or both fibronectin-binding protein genes (27). No *S. aureus* strains have been identified which do not contain either of the fibronectin-binding protein genes (27). On the other hand, data from experimental animal studies demonstrate that the expression of fibronectin-binding proteins is not important for *S. aureus* virulence and may in fact decrease the extent of virulence (8, 15; this study). Interestingly, adhesion of clinical strains of *S. aureus* to fibronectin-coated slides is variable and does not correlate with the number of fibronectin-binding protein genes (27). Some methicillin-resistant *S. aureus* strains are also characterized by their low adherence to fibronectin-coated slides

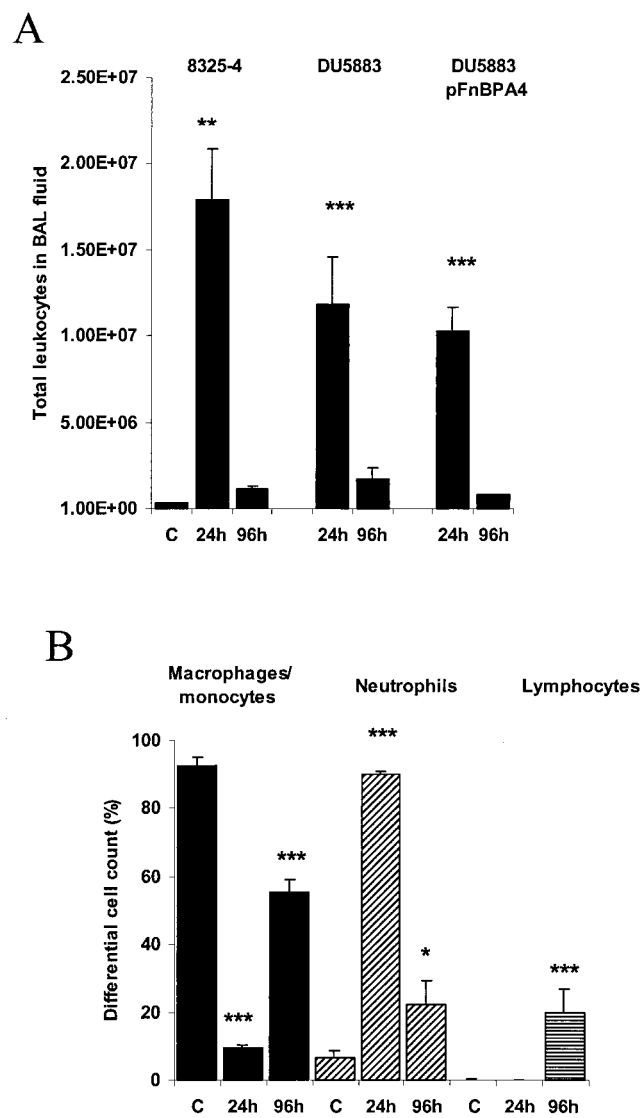


FIG. 6. Number and profile of leukocytes recovered in BAL fluid from *S. aureus*-inoculated lungs at 24 and 96 h postinfection. (A) Total number of leukocytes recovered in BAL fluid from 8325-4-, DU5883- and DU5883(pFnBPA4)-inoculated lungs. (B) Percentage of neutrophils (black bars), macrophages and monocytes (diagonally striped bars), and lymphocytes (horizontally striped bars) in BAL fluid from control and 8325-4-inoculated lungs. \*,  $P < 0.05$  versus control; \*\*,  $P < 0.01$  versus control; \*\*\*,  $P < 0.001$  versus control.

(35). Low adherence of these methicillin-resistant *S. aureus* strains is associated with the expression of a cell surface protein called PIs (plasmin sensitive) (33). It is possible that the antivirulence effects of fibronectin-binding proteins may be masked or modified during infection by the expression of antiadhesion proteins such as PIs.

Integrin antagonists have been proposed as possible new therapeutic agents to aid in the elimination of gram-positive organisms by preventing fibronectin-binding protein-mediated internalization into host cells (6). Our study suggests that such drugs are unlikely to be useful in the treatment of *S. aureus*-induced pneumonia. While we were able to detect

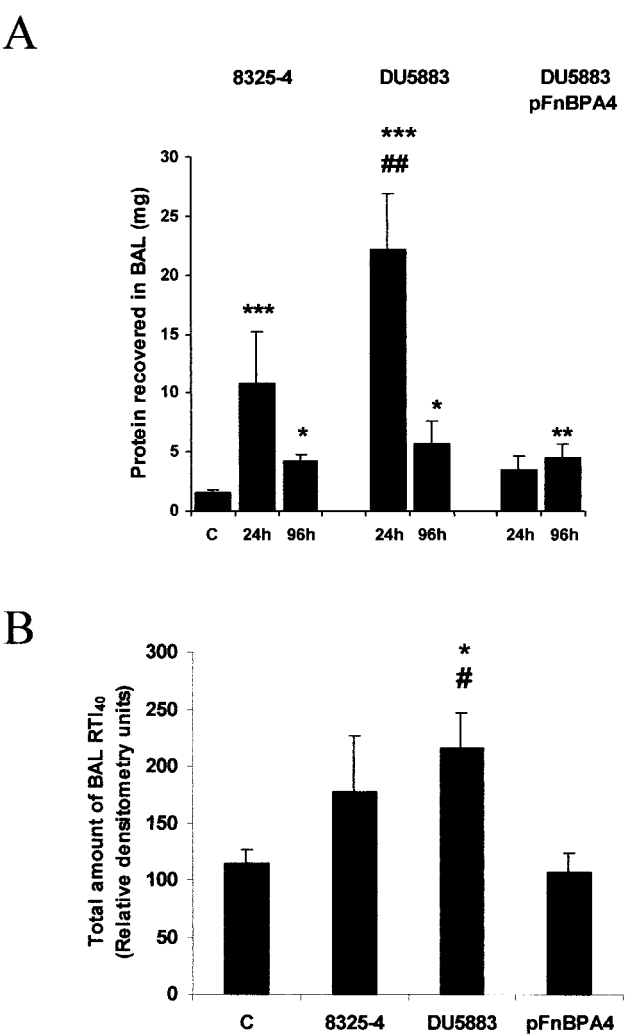


FIG. 7. Amount of protein and RTI<sub>40</sub> recovered in BAL fluid at 24 and 96 h postinfection. (A) The amount of protein recovered in BAL fluid from 8325-4-, DU5883-, and DU5883(pFnBPA4)-inoculated rats at 24 and 96 h postinfection. (B) The amount of RTI<sub>40</sub> recovered in BAL fluid at 24 h postinfection. \*,  $P < 0.05$  versus control; \*\*,  $P < 0.01$  versus control; \*\*\*,  $P < 0.001$  versus control; #,  $P < 0.05$  versus DU5883(pFnBPA4); ##,  $P < 0.01$  versus DU5883(pFnBPA4).

*S. aureus* within neutrophils at 24 h postinfection in rats inoculated with strain 8325-4, we were not able to detect any *S. aureus* internalized into alveolar epithelial cells. Our data suggest that *S. aureus* internalization into alveolar epithelial cells by a fibronectin-binding protein-mediated mechanism is unlikely to be a virulence mechanism in our rat model of pneumonia.

In summary, despite the fact that fibronectin-binding proteins are widely expressed by *S. aureus*, our data suggest that they reduce *S. aureus* virulence in a rat model of pneumonia. Our data raise the possibility that anti-fibronectin-binding proteins, such as PIs, may contribute to virulence by preventing *S. aureus* internalization by both professional and nonprofessional phagocytes.



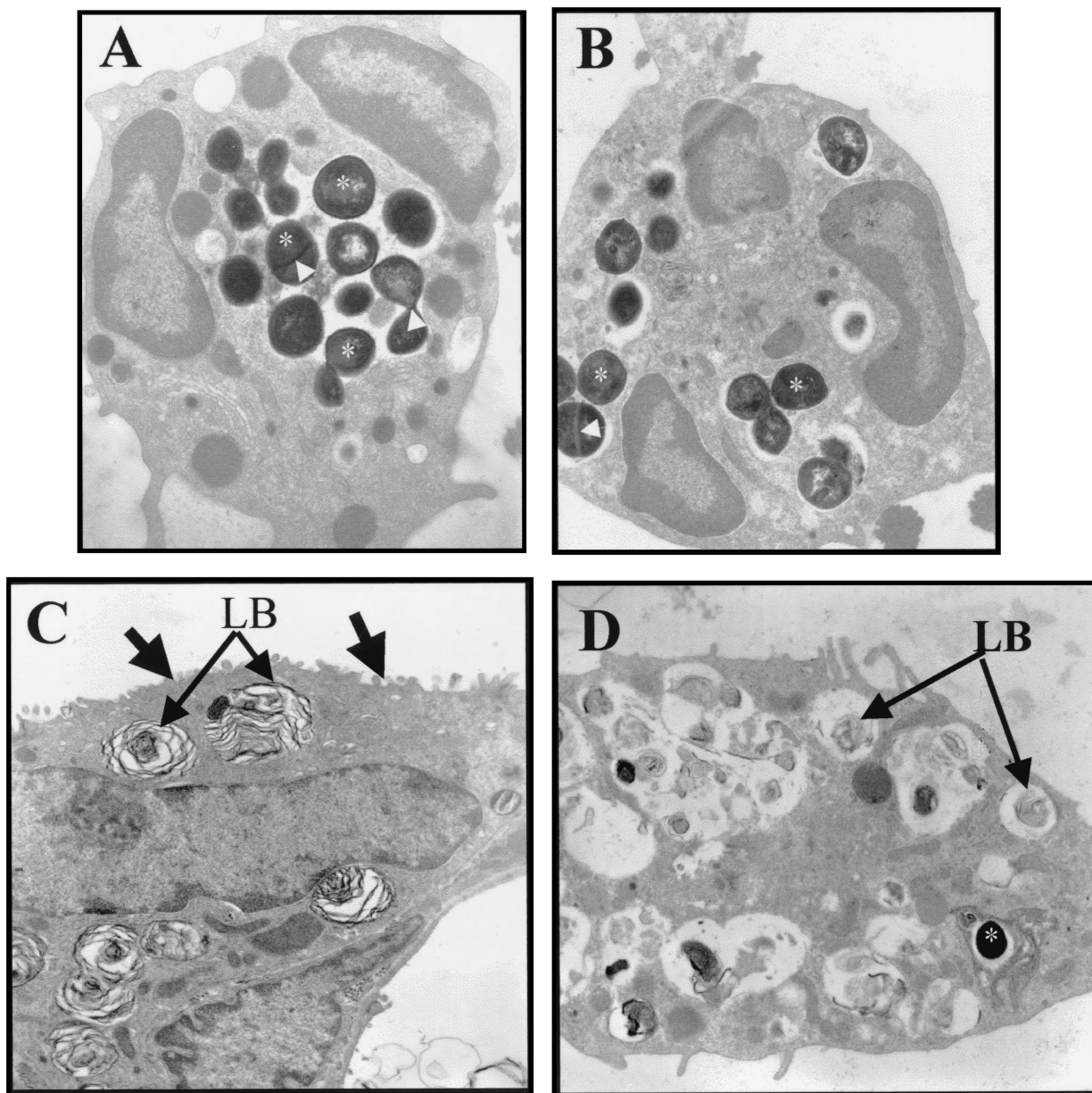


FIG. 8. Cellular location of *S. aureus* at the electron microscopic level in 8325-4- and DU5883-inoculated rats. (A and B) Electron micrograph of neutrophils from 8325-4-infected (A) and DU5883-infected (B) lungs. *S. aureus* (electron dense, approximately 1- $\mu$ m-diameter particles) cells are internalized into neutrophils from both 8325-4- and DU5883-infected lungs (stars on some internalized *S. aureus*). The electron-dense line across some *S. aureus* (arrows in A and B) is a cell wall associated with cell division (37). (C and D) Electron micrograph of an alveolar epithelial type II cell from control (C) and DU5883-inoculated (D) lungs. Type II cell from control lung contains characteristic lamellar bodies (LB) and apical microvilli (arrows) (C). Type II cell from DU5883-infected lung has sloughed from its basement membrane and contains remnants of lamellar bodies (LB) (D). One *S. aureus* bacterium is located in a vacuole within the cytoplasm of the sloughed type II cell (star) (D). Original magnification: A and B,  $\times 13,000$ ; C and D,  $\times 8,000$ .

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